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$CD4^+CD3^-$ Cells Induce Peyer's Patch Development: Role of $\alpha4\beta1$ Integrin Activation by CXCR5

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Summary

$CD4^+CD3^-$ cells are the predominant hematopoietic cells found in mouse fetal intestine. We prove their role as Peyer's patch (PP)-inducing cells by transfer into neonatal PP-deficient mice. To test the requirement of chemokines and adhesion molecules in induction of PP, we studied mice deficient in CXCR5 and/or $\alpha4\beta1$ integrin-mediated adhesion. $CXCR5^{-/-}$ mice have $CD4^+CD3^-$ cells, which are inefficient in inducing PP formation. We show here that CXCR5/CXCL13 signaling activates $\alpha4\beta1$ integrin on $CD4^+CD3^-$ cells. Blocking of $\beta1$ integrin or VCAM-1, the ligand of $\alpha4\beta1$ integrin, inhibits PP formation. This study demonstrates the link between chemokine receptors and adhesion molecules that regulates stromal/hematopoietic cell interaction leading to PP formation.

Introduction

$CD4^+CD3^-$ cells are a unique subset of hematopoietic cells found in spleen, mesenteric lymph node (MLN), and the small intestine (Adachi et al., 1997; Kelly and Scollay, 1992; Mebius et al., 1997). They have emerged as the first hematopoietic cells that home to the gut of fetal mice, and it has been argued that they are responsible for the induction of PP and LN (Adachi et al., 1997; Ansel and Cyster, 2001; Yoshida et al., 1999). Since they are found in Rag-2-deficient mice, they do not require receptor rearrangement (Adachi et al., 1998; Yoshida et al., 1999). They express IL-7Rα (CD127) and LT$\alpha1\beta2$ (Honda et al., 2001; Mebius et al., 1997) and thus are thought to induce ligation and signaling of LTβR on stromal cells, a key step for LN and PP development (Futterer et al., 1998).

PP organogenesis starts around embryonic day E16.5 with the clustering of $LT\beta R^+VCAM\text{-}1^+$ (CD106) $ICAM\text{-}1^+$ (CD54) stromal cells and the subsequent accumulation of $CD4^+CD3^-$ cells at distinct sites along the fetal small intestine (for review see Ansel and Cyster, 2001; Finke and Kraehenbuhl, 2001; Nishikawa et al., 2000). At E18.5, PP and MLN are seeded by the first mature B and T lymphocytes while segregation into follicular B cell and interfollicular T cell regions is not completed before 1 week after birth. Mice deficient for LTα, RORγ, IL-7R, jak3, or Id-2 lack PP and functional $CD4^+CD3^-$ cells (Adachi et al., 1998; De Togni et al., 1994; Sun et al., 2000; Yokota et al., 2000), suggesting that $CD4^+CD3^-$ cells that express these molecules are essential for PP organogenesis. In adult mice, B lymphocytes have been shown to contribute to the maintenance of PP architecture (Debard et al., 2001; Golovkina et al., 1999).

Gene-knockout experiments have revealed a critical role for CXCR5 (BLR1) and its ligand CXCL13 (BLC, BCA-1) in PP development (Ansel et al., 2000; Förster et al., 1996), since in these mice PP are either absent or strongly reduced in number. Stromal cells secrete CXCL13, a chemokine known to recruit B lymphocytes and $IL\text{-}7R\alpha^+$ $CD4^+CD3^-$ cells to lymphoid follicles (Gunn et al., 1998; Honda et al., 2001). Several feedback loops reinforce the interactions between hematopoietic and stromal cells. For example, both CXCL13 and IL-7Rα ligands enhance LT$\alpha1\beta2$ expression on $CD4^+CD3^-$ cells (Ansel et al., 2000; Honda et al., 2001) which in turn triggers LTβR-signaling, VCAM-1 expression, and CXCL13 secretion by stromal cells (Cuff et al., 1999; Honda et al., 2001; Ngo et al., 1999).

Despite the importance of $CD4^+CD3^-$ cells for PP ontogeny, little is known about how these cells establish cellular contacts with intestinal stromal cells. Therefore, it is important to know which chemokines and cell adhesion molecules mediate recruitment and retention of $CD4^+CD3^-$ cells in lymphatic tissue. By E15.5, the addressins VCAM-1, ICAM-1, and MAdCAM-1 are expressed on stromal cells of fetal intestine (Hashi et al., 2001). The high-affinity receptor for MAdCAM-1 is $\alpha4\beta7$ integrin, which mediates lymphocyte binding to high endothelial venules (HEV) in PP and postcapillary venules of the lamina propria (Berlin et al., 1993; Holzmann et al., 1989). $CD4^+CD3^-$ cells isolated from fetal LN and PP express $\alpha4\beta7$ integrin (Mebius et al., 1997). Nevertheless, blocking of $\alpha4\beta7^+$ integrin/MAdCAM-1 interactions by mAb-treatment during embryogenesis only partially affects colonization by fetal $CD4^+CD3^-$ cells, indicating that other addressins such as VCAM-1 could play a role in homing and adhesion of these cells to the lymphoid environment (Mebius et al., 1996).

VCAM-1 is expressed on endothelial cells of peripheral postcapillary venules. Thymic epithelium and bone marrow as well as germinal center stromal cells also express VCAM-1 (Shimizu et al., 1999) that retains leukocytes expressing the $\alpha4\beta1$ integrin (VLA-4; CD49d/CD29) (Larson and Springer, 1990), providing antiapoptotic signals (Hynes and Lander, 1992; Shimizu et al., 1999). In the fetal gut, the role of $\alpha4\beta1$/VCAM-1 has yet not been investigated. The integrin receptor $\alpha4\beta1$ is a heterodimeric transmembrane molecule expressed on hematopoietic cells (Larson and Springer, 1990). $\alpha4\beta1$

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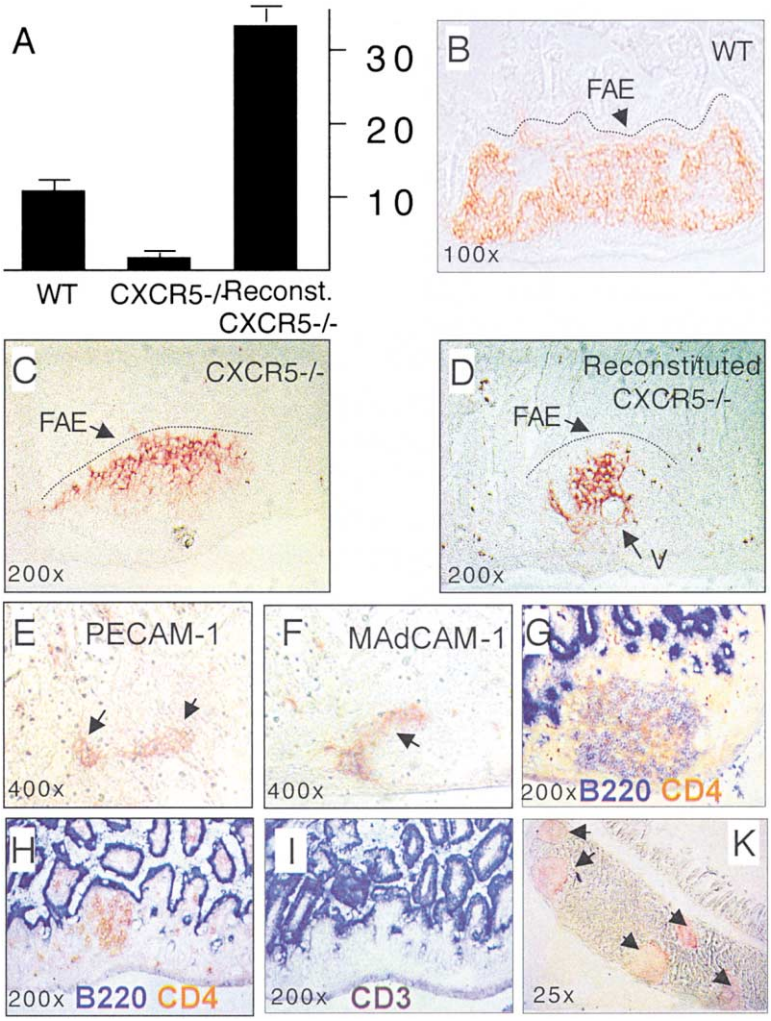

Figure 1. Reconstitution of PP Organogenesis in CXCR5$^{-/-}$ Mice

(A) Numbers of PP in WT mice, CXCR5$^{-/-}$, and CXCR5$^{-/-}$ mice reconstituted with splenic CD4^{+}CD3^{-} cells. Means ± SD of five mice per each group are shown.

(B) Immunohistochemistry with anti-VCAM-1 mAb of PP from 3-week-old C57BL/6 mice. PP stromal cells are specifically stained. The follicle-associated epithelium (FAE) is indicated by a dotted line. Magnification × 100.

(C) Immunohistochemistry of PP from a 3-week-old CXCR5$^{-/-}$ mouse. One out of four detectable PP from whole intestine is shown. The other two mice had no detectable PP. VCAM-1^{+} stromal cells are detectable exclusively in the subepithelial dome. The size of the residual PP is diminished compared to WT mice. Magnification × 200.

(D) 3 weeks after transfer of splenic CD4^{+} CD3^{-} cells isolated from E19.5 embryonic WT mice into newborn CXCR5$^{-/-}$ mice. One out of 33 VCAM-1 clusters from small intestine is shown. VCAM-1 stromal cells are found in close proximity to vascular venules (V). Magnification × 200.

(E and F) HEV are stained with anti-MAdCAM-1 and anti-PECAM-1 mAb on frozen sections of reconstituted CXCR5$^{-/-}$ mice. Magnification × 400.

(G and H) Immunohistochemistry of reconstituted CXCR5$^{-/-}$ mice with anti-B220 (blue) and anti-CD4 (yellow) mAb. Magnification × 200.

(I) Anti-CD3 staining (blue) of consecutive sections reveals the absence of CD3^{+}T cells in reconstituted CXCR5$^{-/-}$ mice. Magnification × 200.

(K) VCAM-1 expression in CXCR5$^{-/-}$ mice 3 weeks after transfer of 10^{6} total WT fetal spleen cells Magnification × 25.

integrin plays an essential role in a variety of biological processes ranging from cell migration to tissue organization, growth, survival, and differentiation (Hynes and Lander, 1992; Shimizu et al., 1999; Finke and Acha-Orbea, 2001; Finke et al., 2001). Integrin function is regulated by alteration of affinity for extracellular ligands, a mechanism known to be dependent on activation and conformational change of the dimeric integrin molecule (Hynes and Lander, 1992; Diamond and Springer, 1994; Schwartz et al., 1995). Alternatively, changes in integrin mobility and clustering can contribute to enhanced adhesion (Yauch et al., 1997). The affinity modulation (referred to as "inside-out signaling") of integrins for their ligands is dynamically regulated by signals received from the local environment through cell surface receptors. These include T and B cell receptors, cytokine receptors, costimulatory molecules, and chemokine receptors (Kovach et al., 1995; Schwartz et al., 1995; Fenczik et al., 1997; Giblin et al., 1997; Peled et al., 2000). Previous work established that cells modulate the affinity of $\alpha4\beta1$ integrin for VCAM-1 (Rose et al., 2000; Finke and Acha-Orbea, 2001). Here, we demonstrate that fetal CD4^{+}CD3^{-} hematopoietic cells induce PP formation in CXCR5$^{-/-}$ mice. Moreover, activated $\alpha4\beta1$ integrin is expressed on all fetal CD4^{+}CD3^{-} cells together with $\alpha4\beta7$ integrin in wild-type (WT) mice. We show that CXCR5$^{-/-}$ mice have even increased numbers of CD4^{+}CD3^{-} hematopoietic cells. However, these cells lack activated $\beta1$ integrin in PP, peripheral LN, and spleen. Blocking of $\alpha4\beta1$ integrin-VCAM-1 interaction during fetal development allows entry of CD4^{+}CD3^{-} cells into the gut, but PP formation is strongly inhibited. Therefore, an important feedback loop among CXCL13-secreting stromal cells and CXCR5^{+} CD4^{+}CD3^{-} cells has been established resulting in activation of $\beta1$ integrin and anchoring to intestinal stromal cells. This is crucial for PP development, allowing continuous LTβR-signaling in stromal cells.

Results

Wild-Type CD4^{+}CD3^{-} Cells Induce PP Formation in CXCR5$^{-/-}$ Mice

To formally prove the role of CD4^{+}CD3^{-} cells as inducers of PP, we transferred 2×10^{4} WT fetal spleen-derived CD4^{+}CD3^{-} cells i.p. into newborn CXCR5$^{-/-}$ mice. Two to six weeks later, the number of PP in the gut of recipient mice was determined. The formation of PP was monitored by anti-VCAM-1 immunohistochemistry on serial sections from total small intestine, as it allowed detec-

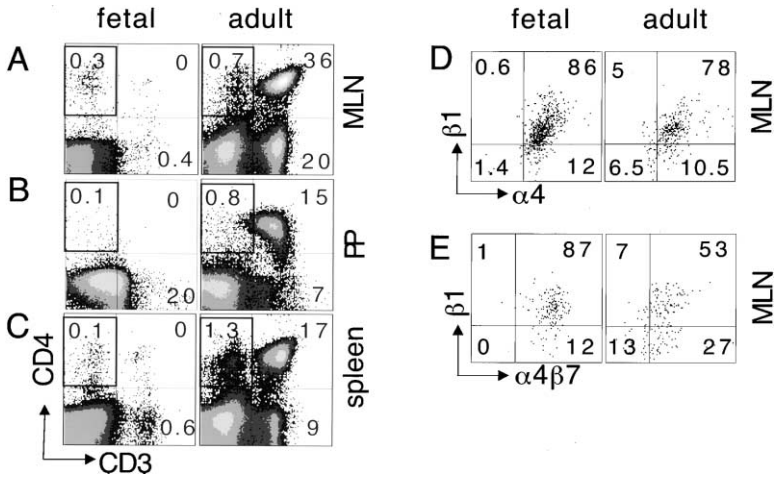

Figure 2. $CD4^+CD3^-$ Cells Coexpress $\alpha4\beta1$ and $\alpha4\beta7$ Integrin

(A–C) Flow cytometry analysis of E18.5-d1 (left) and adult (right) MLN (A), PP (B), and spleen (C) from C57BL/6 mice. $CD4^+CD3^-$ cells can be detected in both fetal and adult organs.

(D) Surface expression of total β1 integrin (HA25) and α4 integrin (PS/2) on cells gated on $CD4^+CD3^-$ of fetal and adult MLN. The majority of cells from both ages coexpress β1 and α4 integrins.

(E) Expression of β1 integrin (HA25) and $\alpha4\beta7$ integrin (DATK 32) on cells gated for $CD4^+CD3^-$ from fetal and adult MLN. The majority of cells from both ages coexpress β1 and $\alpha4\beta7$ integrins.

tion of even very small PP. In control WT mice, we observed ten to eleven large PP with homogenous expression of VCAM-1 (Figures 1A and 1B), whereas in $CXCR5^{-/-}$ mice two out of three mice had no detectable PP and in one out of three mice four PP were found (Figures 1A and 1C). Transfer of $CD4^+CD3^-$ cells into newborn $CXCR5^{-/-}$ mice dramatically increased the number of PP (mean value 33) (Figure 1A). The newly formed PP-like follicles were localized on the antimesenteric site of the small intestine, and their organization was similar to that of WT PP with typical $MAdCAM\text{-}1^+$ and $PECAM\text{-}1^+$ HEV and a network of $VCAM\text{-}1^+$ stromal cells and $CD4^+CD3^-$ cells (Figures 1D–1F and 1H). $B220^+$ B cells but not $CD3^+$ cells were occasionally detected in the PP of reconstituted mice (Figures 1G and 1I). This is most likely due to lack of CXCR5 on mature lymphocytes of recipient mice. By reconstituting mice with total WT spleen cells, large $VCAM\text{-}1^+$clusters were generated (Figure 1K), suggesting that donor-derived splenic lymphocyte precursors could compensate for the lack of CXCR5 on recipient lymphocytes. Adoptive transfer of 10^6 sorted B cells isolated from adult WT mice, WT $IL\text{-}7R\alpha^+$ fetal liver cells, $CXCR5^{-/-}$, $IL\text{-}7R\alpha^{-/-}$, or $LT\alpha^{-/-}$ total spleen cells was unable to trigger VCAM-1 spots (data not shown). Taken together, these data demonstrate the striking in vivo capacity of fetal WT $CD4^+CD3^-$ cells to restore defective PP formation in newborn CXCR5-deficient mice.

$CD4^+CD3^-$ Cells Express $\alpha4\beta1$ and $\alpha4\beta7$ Integrin

By comparing fetal and adult mice between 0.3%–0.7% $CD4^+CD3^-$, cells were found among all leukocytes of fetal and adult MLN (Figure 2A). We did not discriminate between $CD45^+$ and $CD45^-$ cells since fetal $CD4^+CD3^-$ cells express low levels of CD45. As MLN cannot be dissected completely free of surrounding tissue when isolated from E18.5 embryos, the percentage of cells from fetal MLN most likely represents an underestimate. Similar percentages were found in PP (Figure 2B) and spleen, the latter harboring predominantly $CD4^{low}CD3^-$ cells (Figure 2C). $CD4^+CD3^-$ cells are localized in the developing gut in close vicinity to VCAM-1-expressing stromal cells (Adachi et al., 1997; Honda et al., 2001; Yoshida et al., 1999), and they express $LT\alpha1\beta2$ which can crosslink LTβR on stromal cells. This is a key interaction in LN and PP development as well as in maintenance of splenic microarchitecture. Signal transduction on fetal stromal cells requires cellular cross-talk via soluble factors and/or cellular adhesion. In order to study how $CD4^+CD3^-$ cells interact with $VCAM\text{-}1^+$ stromal cells, we analyzed expression of $\alpha4\beta1$ integrin, the high-affinity receptor for VCAM-1 (Chan et al., 1992; Elices et al., 1990). Independent mAb stainings for α4 and β1 integrin reveals coexpression of both integrin subunits on 86% of $CD4^+CD3^-$ cells from fetal, and 78% of $CD4^+CD3^-$ cells from adult MLN, respectively (Figure 2D). Flow cytometry analysis revealed that fetal and adult mice harbor $CD4^+CD3^-$ cells coexpressing β1 integrin and $\alpha4\beta7$, the integrin receptor for MAdCAM-1 and the low-affinity receptor for VCAM-1 (Figure 2E). Similar results were obtained from analyzing E18.5 neonatal and adult PP (data not shown).

Activation of β1 Integrin on Intestinal $CD4^+CD3^-$ Cells Is Regulated by CXCR5

Integrin-mediated adhesion is dependent on the activation of the heterodimer inducing a conformational change, which enhances the affinity for its ligand (Hynes, 1992). In order to test the functional activity of β1 integrin, intestinal $CD4^+CD3^-$ cells were analyzed by flow cytometry using the anti-murine β1 integrin mAb 9EG7 which is specific for an activation-dependent epitope (Lenter et al., 1993). 84% of $CD4^+CD3^-$ cells isolated from fetal MLN and 79% of the cells isolated from adult MLN express β1 integrin in its activated conformation (Figure 3A). The majority of adult $CD3^+$ T cells did not express β1 integrin (5%), while 64% of fetal $\gamma\delta^+CD3^+$T cells were β1 $integrin^{high}$. This demonstrates a dramatic activation of β1 integrin on $CD4^+CD3^-$ cells in fetal and adult mice. Integrins can be activated in response to cytoplasmatic signals initiated through the activation of other cellular receptors such as chemokine receptors (Butcher, 1991; Campbell et al., 1996; Mackay, 2001). The expression of CXCR5 and CCR7 on $CD4^+CD3^-$ cells of fetal MLN has been documented (Honda et al., 2001; Mebius et al., 1997). In adult WT mice, the large majority of β1 integrin (80%) is expressed in its activated form in MLN (Figure 3B). In $CXCR5^{-/-}$ mice, $CD4^+CD3^-$ cells

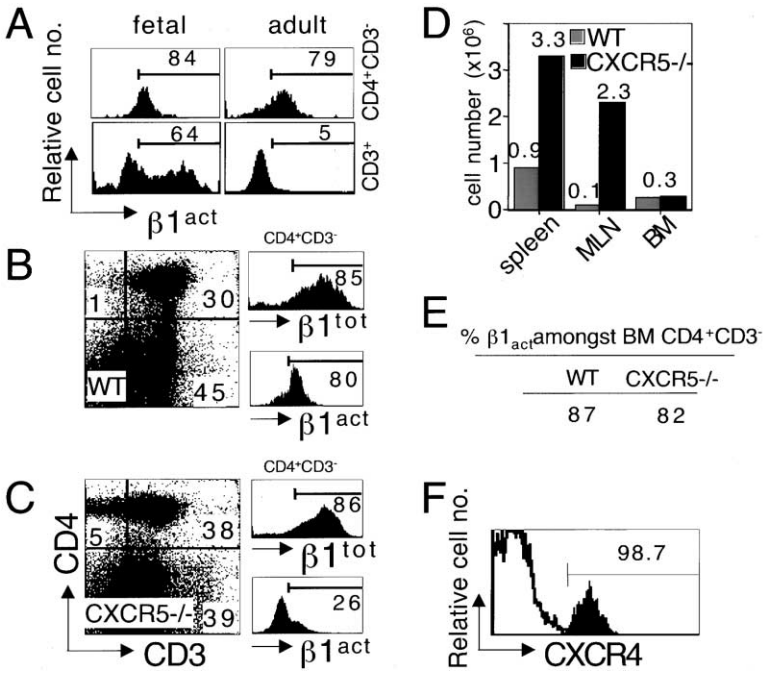

Figure 3. CXCR5 Regulates Activation of β1 Integrin in the Periphery

(A) Flow cytometry analysis of fetal (left) and adult (right) MLN gated on $CD4^+CD3^-$ cells (filled histogram) stained with 9EG7 mAb specific for β1 integrin in activated form. As control, expression levels of activated β1 integrin on fetal and adult $CD3^+$cells are shown.

(B) Flow cytometry analysis of MLN of 4-week-old C57BL/6 mice. Gated on $CD4^+CD3^-$ cells expression of total or activated β1 integrin is shown by histogram (right).

(C) Flow cytometry analysis of MLN of 4-week-old $CXCR5^{-/-}$ mice. Gated on $CD4^+CD3^-$ cells expression of total or activated β1 integrin is shown by histogram (right).

(D) Mean number of $CD4^+CD3^-$ cells in spleen, MLN, and BM of WT and $CXCR5^{-/-}$ mice (three mice each). Differences in cell numbers between $CXCR5^{-/-}$ and WT mice are statistically significant in spleen and MLN ($p < 0.005$).

(E) Flow cytometry as in (C) was used to identify $\beta1_{act}$ integrin-expressing cells among $CD4^+CD3^-$ from BM of WT and $CXCR5^{-/-}$ mice.

(F) CXCR4 expression on fetal $CD4^+CD3^-$ cells (filled histogram). As control, nonhematopoietic cells are shown (overlay, transparent). Data are representative of four experiments with two to three mice in each group.

are detectable by flow cytometry in freshly isolated MLN, bone marrow (BM), and spleen (Figures 3C and 3D) as well as in the gut (data not shown). Although among these cells expression levels of total β1 integrin (detectable by mAb HA2/5) are comparable to control mice (85% in WT, 86% in $CXCR5^{-/-}$), they show strongly reduced expression levels (26%) of activated β1 integrin (compare Figures 3B and 3C, right). Taken together, $CD4^+CD3^-$ cells of $CXCR5^{-/-}$ mice have a defect in activation of β1 integrin in secondary lymphoid organs.

Surprisingly, the percentage of $CD4^+CD3^-$ cells in MLN is five times higher in $CXCR5^{-/-}$ mice (5%) than in WT mice (1%), while CD3 T cell percentages are unaffected (77% in $CXCR5^{-/-}$ as compared to 75% in WT mice) (Figures 3B and 3C). This is even more striking when absolute cell numbers are compared (Figure 3D). In adult WT mice, 0.1×10^6 $CD4^+CD3^-$ cells are detectable, whereas in $CXCR5^{-/-}$ mice, 2.3×10^6 $CD4^+CD3^-$ cells are found in MLN. In spleen, we found 3.3×10^6 $CD4^+CD3^-$ cells compared to 0.9×10^6 $CD4^+CD3^-$ cells in WT mice. In fetal mice, there was a 10-fold increase in MLN $CD4^+CD3^-$ cell numbers (data not shown). Taken together, a dramatic accumulation of $CD4^+CD3^-$ cells lacking activated α4β1 integrin is observed in MLN and spleen of $CXCR5^{-/-}$ mice. Therefore, migration to MLN and retention of these hematopoietic cells in MLN and spleen seems to be independent of functional CXCR5 and/or activated β1 integrin. Interestingly, in BM from WT or $CXCR5^{-/-}$ mice the percentages and absolute cell numbers of $CD4^+CD3^-$ cells are identical (Figure 3D). Moreover, the percentage of activated β1 integrin on BM $CD4^+CD3^-$ cells of $CXCR5^{-/-}$ mice (82%) is as high as in WT animals (87%) (Figure 3E), suggesting that activation of β1 integrin is differentially regulated in the BM and periphery. It has been reported that the chemokine CXCL12 secreted by BM stromal cells activates integrins such as LFA-1 (CD11a) and β1 integrin (Peled et al., 2000). In agreement with this, we found the corresponding chemokine receptor CXCR4 highly expressed on $CD4^+CD3^-$ cells (Figure 3F). The degree of expression is comparable in MLN, spleen, and BM independent of age (data not shown). These data provide further evidence that expression of multiple chemokine receptors such as CXCR5 and CXCR4 determine whether or not inside-out signals to β1 integrin activation can be provided. The limiting factor, however, seems to be the availability of the corresponding chemokines since only the BM environment might compensate for the lack of CXCR5.

β1 Integrin$^+$/VCAM-1 Adhesion Is Essential for Induction of PP Organogenesis by $CD4^+CD3^-$ Cells

Based on the observation that activated β1 integrin is expressed on WT $CD4^+CD3^-$ cells but highly reduced in $CXCR5^{-/-}$ secondary lymphoid organs, we examined the effect of α4β1 integrin inhibition on PP development by injecting blocking mAb during embryogenesis. Pregnant mice were injected i.p. with 500 μg of anti-$\beta1$ integrin^{act} mAb (9EG7) or anti-VCAM-1 mAb (MK2.7) at E12.5 and subsequently every 3 days until birth. As previously shown, both mAbs efficiently block integrin-mediated adhesion in vivo (Finke and Acha-Orbea, 2001). Immunohistochemical examination of total intestine from day 2 offspring revealed a statistically significant reduction in numbers of PP from 10.3 ± 1.3 to 7.9 ± 2 in the small intestine ($p < 0.05$) (Figure 4A). Moreover, the remaining PP were significantly smaller (Figure 4B). This inhibitory effect on PP formation was also evident when anti-VCAM-1 mAb was administered (7.7 PP ± 0.6; $p < 0.05$). Therefore, cellular adhesion of β1 integrin$^+$$CD4^+CD3^-$

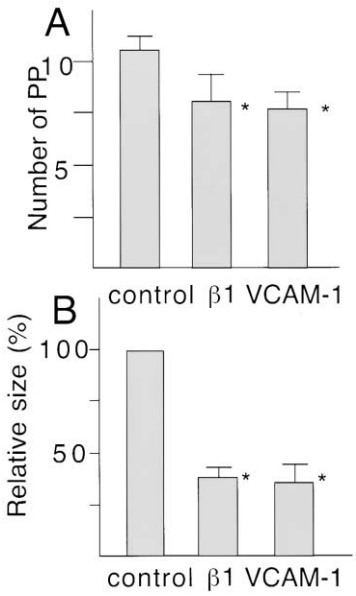

Figure 4. PP Development in Mice after Inhibition of Fetal β1 Integrin/VCAM-1-Mediated Cell Adhesion

(A) Whole intestines of control mice or mice treated from E12.5 with anti-β1 integrin Ab (9EG7) or anti-VCAM-1 Ab (MK 2.7) were removed 2 days after birth, and the numbers of PP were determined by immunohistochemistry with anti-VCAM-1 mAb. In normal small intestine, a mean of 10.3 PP was found. An asterisk denotes differences between Ab-treated mice and control mice which are statistically significant ($p < 0.05$; Student's t test).

(B) Relative size of PP was determined by calculating the numbers of pixels per surface of immunostained sections of PP derived from mAb-treated mice compared to surface of PP from control mice (estimated as 100%) using the Adobe Photoshop 5.5 software.

cells to intestinal VCAM-1$^+$ stromal cells is indispensable for maturation and formation of PP architecture. Importantly, we obtained similar results when CD4$^+$CD3$^-$ cells were totally depleted from the pool of circulating cells by injecting anti-CD4 mAb (GK 1.5) during embryonic development (data not shown), emphasizing the key role of CD4$^+$CD3$^-$ cells in PP development.

β1 Integrin on CD4$^+$CD3$^-$ Cells Is Not Necessary for Homing to Intestine

We analyzed whether blocking α4β1 integrin or its ligand VCAM-1 had an effect on CD4$^+$CD3$^-$ cell migration during fetal development. Following the protocol described before, pregnant mice were repeatedly injected with anti-integrin mAb starting from E12.5. Immunohistochemical analysis of PP, MLN, and spleen in the offspring 2–6 days after birth revealed a 3- to 4-fold reduction in the size of all lymphoid organs of mice treated with anti-β1 integrin mAb (Figure 5). The number of B220$^+$ B cells and CD3$^+$ T cells was dramatically reduced in residual PP, MLN, and spleen (Figure 5). This is in agreement with previous reports demonstrating that β1 integrin expression is a prerequisite for efficient migration of hematopoietic precursor cells to fetal liver, spleen, and BM (Fässler and Meyer, 1995; Papayannopoulou et al., 1995; Hirsch et al., 1996; Potocnik et al., 2000; Williams et al., 1991). The absolute cell number of CD4$^+$CD3$^-$ cells in PP, spleen, and MLN of anti-β1 integrin-treated mice was determined by immunohistochemistry (Figure 5) and flow cytometry (data not shown) and was comparable to control mice, indicating that migration of CD4$^+$CD3$^-$ cells occurs independently of β1 integrin function.

Clustering of VCAM-1$^+$ Cells Is Dependent on Activated β1 Integrin

One of the earliest events in PP anlage formation is the clustering of VCAM-1$^+$ cells in segregated cellular compartments (Hashi et al., 2001). We addressed the role of CD4$^+$CD3$^-$ cells in the induction of cluster formation of VCAM-1-expressing stromal cells in the small intestine. We analyzed whole mount intestine of d2 WT mice (Figure 6A) or serial sections of small intestine from CXCR5$^{-/-}$ mice which still can form residual PP (Figure 6B) and LTα$^{-/-}$ mice completely lacking PP (Figure 6C) by immunostaining with anti-VCAM-1 mAb. In CXCR5$^{-/-}$ mice and LTα$^{-/-}$ mice, VCAM-1 clusters assembling in PP anlage were rarely or never observed, respectively. In the villi, single VCAM-1$^+$ cells were distributed all over the small intestine (Figures 6B and 6C, arrows). They were clearly distinguishable from basal PP-like structures in size, cellularity, and vascularization. To address the role of activated β1 integrin in this process, we treated mice during embryogenesis with Abs specific for the activated form of β1 integrin (9EG7) (Figure 6D). Similar to the KO mice, antibody-treated mice showed reduced numbers of small clusters but high numbers of VCAM-1$^+$ cells scattered all over the small intestine. Taken together, these results suggest that signaling through activated β1 integrin triggers cluster formation, an initial step that allows CXCR5, IL-7Rα, and LTβR-mediated cross-talks between hematopoietic and stromal cells followed by PP formation.

Discussion

PP organogenesis is initiated at sites where LTβR$^+$VCAM-1$^+$ stromal cells are found (Adachi et al., 1997). CD4$^+$CD3$^-$ hematopoietic cells have been proposed as essential partners of LTα1β2/LTβR-mediated signals inducing PP development (Yoshida et al., 1999). In this study, we established the role of these cells in PP induction by their adoptive transfer into neonatal mice deficient in PP. We also characterized the mechanism of cellular adhesion between CD4$^+$CD3$^-$ cells and VCAM-1$^+$ stromal cells via preactivated β1. Finally, we characterized a loop provided by CXCL13/CXCR5 interaction that activates the β1 integrin on CD4$^+$CD3$^-$ cells and showed that this integrin-mediated adhesion allows information exchange necessary for PP induction.

Analysis of LN and PP development in mice reveals that feedback loops among receptor/ligand pairs play an essential role in mediating tissue differentiation. For example, both CXCL13 and IL-7 can induce LTα1β2 expression (Ansel et al., 2000; Nishikawa et al., 2000). In turn, signaling via the LTα1β2-LTβR pathway further enhances the production of chemokines and adhesion molecules (Cuff et al., 1999; Ngo et al., 1999), that are

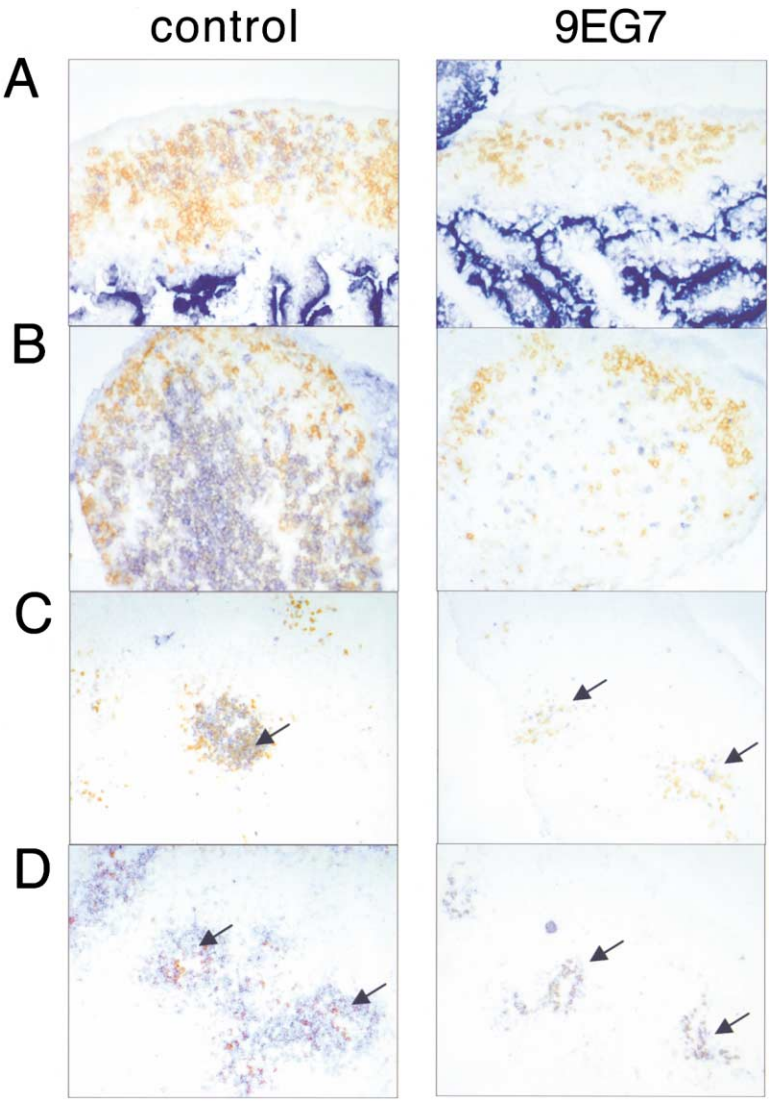

Figure 5. Effect of Anti-β1 Integrin Treatment during Fetal Development on Tissue Distribution of $CD4^+CD3^-$ Cells

(A) Immunohistochemistry of PP from d2 BALB/c mice (left) and d2 BALB/c mice treated from E12.5 with repeated injections of neutralizing anti-$\beta1^{act}$ integrin Ab (9EG7) (right). Double staining with anti-CD4 (yellow) and anti-CD3 (blue) is shown. Magnification × 200.

(B) Double staining with anti-CD4 (yellow) and anti-CD3 (blue) of LN from d2 BALB/c (left) and anti-β1 integrin treated d2 mouse (right) is shown. Magnification × 200.

(C) Double staining with anti-CD4 (yellow) and anti-CD3 (blue) of spleen from d2 BALB/c (left) and anti-β1 integrin treated d2 mouse (right) is shown. Magnification × 100.

(D) Double staining with anti-CD4 (brown) and anti-B220 (blue) of spleen from d2 BALB/c (left) and anti-β1 integrin treated d2 mouse (right) is shown. Arrows indicate central arteria. Magnification × 100.

crucial for the formation of PP anlage, provided that β1 integrin becomes activated as shown in this study.

The observation that $CD4^+CD3^-$ cells express activated β1 integrin raises the possibility that this integrin has an essential function in $CD4^+CD3^-$ cell migration to the developing gut and in cellular attachment to $VCAM\text{-}1^+$ stromal cells. Previously, we have identified the activated form of β1 integrin as an important adhesion molecule expressed by activated B lymphocytes allowing plasmablasts to migrate and home to non-lymphoid tissue (Finke et al., 2001). Moreover, β1 integrin is crucial for adhesion of hematopoietic stem cells to the vascular endothelium in hematopoietic organs (Fässler and Meyer, 1995; Hirsch et al., 1996; Potocnik et al., 2000). Therefore, blocking β1 integrin during embryogenesis should result in a reduction of all hematopoietic cell types in the periphery. In this study, however, anti-β1 integrin-treatment inhibited colonization of peripheral lymphoid organs with B and T lymphocytes (see Figure 5) but not with $CD4^+CD3^-$ cells, as reflected by their presence in LN, spleen, intestine, and bone marrow. It could be that high levels of β1 integrin expression on $CD4^+CD3^-$ cells were sufficient to overcome the effect of anti-β1 integrin mAbs. Alternatively, $CD4^+CD3^-$ cells use different migration pathways and/or adhesion molecules other than β1 integrin to exit the pool of circulating cells. Consistent with this, we and others observed high expression levels of α4β7 integrin in fetal $CD4^+CD3^-$ cells of spleen and LN (Iizuka et al., 2000; Mebius et al., 1996). α4β7 integrin can bind to MAdCAM on HEV of fetal LN and trigger entry of hematopoietic cells into the developing gut. It is possible that α4β7 integrin mediates adhesion of $CD4^+CD3^-$ cells to intestinal HEV and entry of the cells into the gut. However, injecting anti-α4β7 mAb could only partially block the homing of $CD4^+CD3^-$ cells to the gut, indicating that additional adhesion molecules are notably involved in extravasation and homing (Mebius et al., 1996). It remains to be investigated how $CD4^+CD3^-$ cells enter the embryonic gut and whether or not entry depends on the embryonic vascular system. Apparently, β1-mediated adhesion is dispensable for $CD4^+CD3^-$ cell colonization of fetal intestine. In contrast, the number and size of PP is significantly reduced when β1 integrin/VCAM-1

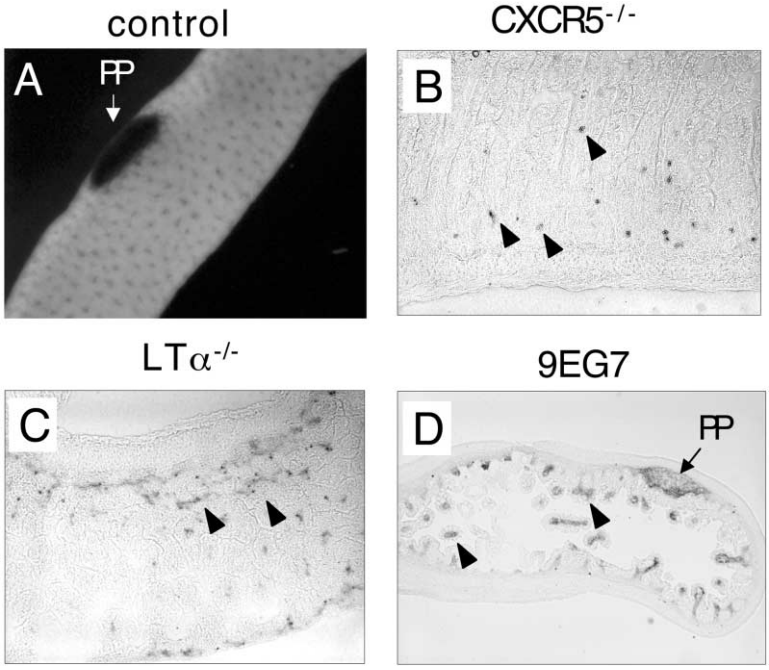

Figure 6. Role of $CD4^+CD3^-$-Mediated Signals in Clustering of $VCAM\text{-}1^+$ Intestinal Stromal Cells

(A) Whole mount immunohistochemistry of d2 small intestine from C57BL/6 mouse stained with anti-VCAM. Compartmentalization of $VCAM\text{-}1^+$ cells forming PP anlage can be distinguished from scattered $VCAM\text{-}1^+$ cells all over the gut.

(B) Immunohistochemistry with anti-VCAM-1 mAb on frozen section of $LT\alpha^{-/-}$ mice. Arrows indicate scattered single $VCAM\text{-}1^+$ cells in the lamina propria. Magnification × 200.

(C) Immunohistochemistry with anti-VCAM-1 mAb on frozen section of $CXCR5^{-/-}$ mice. Arrows indicate scattered single $VCAM\text{-}1^+$ cells in the lamina propria. Magnification × 200.

(D) Immunohistochemistry with anti-VCAM-1 mAb on frozen section of anti-β1 activated (9EG7)-treated mice. Arrows indicate one PP and scattered single $VCAM\text{-}1^+$ cells in the lamina propria. Magnification × 100.

interaction is inhibited during embryogenesis (Figure 4) but not following anti-α4β7 integrin mAb-treatment. Moreover, β7-deficient mice have normal numbers of PP (Wagner et al., 1996). Consistent with this report, we demonstrate that activated α4β1 integrin but not α4β7 is required for PP organogenesis. Together with the finding that $\alpha4\beta1^+CD4^+CD3^-$ cells are the unique source of LTα1β2 in embryonic intestine, our data indicate an essential role for β1 integrin-mediated anchoring of $CD4^+CD3^-$ cells to intestinal $VCAM\text{-}1^+$ stromal cells, thus suggesting an interaction that is required for LTβR signaling and PP formation.

The function of adhesion molecules can be modulated by conformational changes of the corresponding integrin receptor that results in enhanced affinity (van Kooyk and Figdor, 2000). Many external stimuli such as cytokines and chemokines have been shown to activate integrins via an inside-out signal such as activation of the LFA complex by TCR-CD3. $VCAM\text{-}1^+$ stromal cells in fetal intestine produce CXCL13, a chemokine known to direct B cells to lymphoid follicles (Gunn et al., 1998; Honda et al., 2001). $CD4^+CD3^-$ cells express the cognate CXCR5 receptor, and in vitro data indicate that they can respond to CXCL13 (Honda et al., 2001). Based on these studies, we tested whether CXCL13 triggers activation of β1 integrin on $CD4^+CD3^-$ cells. Here, we show that in CXCR5-deficient mice most of β1 integrin is expressed in its nonactivated conformation on cells isolated from spleen or MLN (Figure 3C), while it is activated on BM-derived cells (Figure 3E). In addition, $CD4^+CD3^-$ cells are accumulating in MLN and spleen but not in BM (Figure 3D). This could either reflect influx of resting cells or be a result of cell divisions. We favor the last interpretation, since we observed increasing numbers of proliferating $CD4^+CD3^-$ cells in $CXCR5^{-/-}$ mice compared to WT animals (our unpublished data). These data strongly suggest that the affinity of β1 integrin is differentially regulated in peripheral lymphoid organs (spleen, MLN, PP) and BM. Other chemokines or cytokines such as CXCL12 (SDF-1), stem cell factor (SCF), and Mip-1α (CCL3) are known to be implicated in α4β1 integrin activation (Campbell et al., 1996; Kovach et al., 1995; Peled et al., 2000). Since β1 integrin is activated in BM-derived $CD4^+CD3^-$ cells, CXCL12 and/or SCF secretion by BM stromal cells may be sufficient to trigger integrin-activation. Peripheral CXCL12 secretion levels, however, seem to be too low to completely compensate for the lack of CXCR5/CXCL13 signaling in spleen and LN. This is in agreement with the finding that CXCL12 levels in the liver decrease as the BM becomes available for hematopoietic progenitor cells colonization during embryonic development (McGrath et al., 1999). Therefore, CXCL12 could preferentially mediate BM localization of $CD4^+CD3^-$ cells as shown for other hematopoietic cell populations (Shen et al., 2001). The defect of PP development in $CXCR5^{-/-}$ mice is incomplete since on average two PP are found in their gut. As shown in Figure 3C, activated β1 integrin was strongly reduced but not completely abolished in $CXCR5^{-/-}$ mice. This confirms our hypothesis that alternative chemokines such as CXCL12 have the potential to activate α4β1 integrin, although this pathway seems inefficient in the periphery. We have identified the feedback loop in peripheral lymphoid organs between CXCR5 and β1 integrin. Conceivably, the coupling of CXCR5 to β1 integrin-activation allows differential regulation of adhesive versus chemoattractant responses during migration and positioning of the $CD4^+CD3^-$ cells in the developing gut.

In $CXCR5^{-/-}$ mice, PP development is restored when fetal WT $CD4^+CD3^-$ cells are transferred into newborn $CXCR5^{-/-}$ mice. In contrast, adult B cells or fetal spleen cells derived from $IL\text{-}7R\alpha^{-/-}$ or $LT\alpha^{-/-}$ mice did not

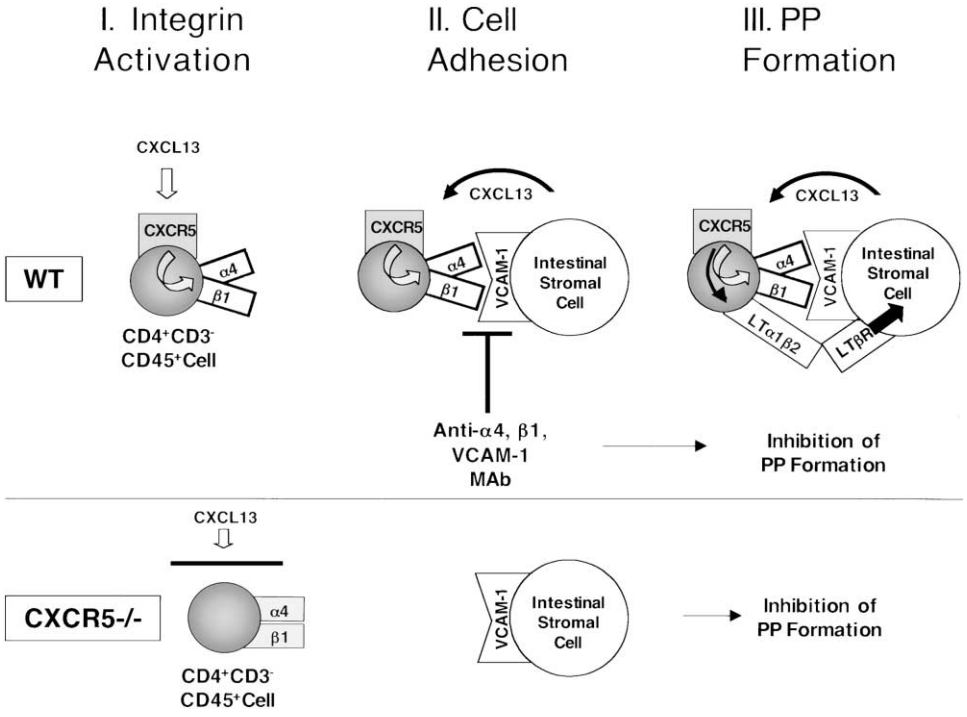

Figure 7. Model of PP Development

(I) Integrin activation. In WT mice, $\alpha4\beta1$ integrin expressed on peripheral $CD4^+CD3^-$ cells becomes activated via CXCL13/CXCR5 signaling in the periphery. In $CXCR5^{-/-}$ mice, activation of $\alpha4\beta1$ is insufficient.

(II) Cell adhesion. Activated $\alpha4\beta1$ integrin mediates adhesion of $CD4^+CD3^-$ cells to $VCAM\text{-}1^+$ intestinal stromal cells, which secrete CXCL13 and therefore can maintain activation of $\alpha4\beta1$ integrin and cellular attachment. $CD4^+CD3^-$ cells isolated from $CXCR5^{-/-}$ mice or animals treated with mAb (anti-$\alpha4$, -$\beta1$, -VCAM-1) fail to adhere to intestinal stromal cells.

(III) Cellular attachment allows continuous signaling via $LT\alpha1\beta2/LT\beta R$ and differentiation of stromal cells. In addition, CXCR5 and IL-7R-signaling enhances expression of $LT\alpha1\beta2$. In Ab-treated or $CXCR5^{-/-}$ animals, PP formation is inhibited.

trigger formation of VCAM-1 spots, indicating a unique role of $CD4^+CD3^-$ cells expressing IL-7Rα and $LT\alpha1\beta2$. A striking feature of the newly generated PP in reconstituted $CXCR5^{-/-}$ mice is the clustering of $VCAM\text{-}1^+$ stromal cells around postcapillary venules and the formation of MadCAM-1^+ HEV (Figure 1). It is likely that circulating $CD4^+CD3^-$ cells migrate along CXCL13 gradients secreted by $VCAM\text{-}1^+$ cells (Honda et al., 2001) and initiate clustering of $VCAM\text{-}1^+$ stromal cells at sites of extravasation in newborn gut. Of note is the fact that we were unable to restore development of inguinal LN. Therefore, organogenesis of lymphatic tissue is orchestrated by a complex interplay of signaling ligand/receptor pairs which differ between peripheral LN and PP (Kim et al., 2000).

It has been reported that single IL-$7R\alpha^+$ cells are found in the developing gut as early as E13.5 before the first VCAM-1 clusters are observed (E15.5) (Adachi et al., 1997; Yoshida et al., 1999). Thereafter, IL-$7R\alpha^+$ $CD4^+CD3^-$ cells appear as aggregated cell clusters in the E16.5 gut. We show here that $LT\alpha^{-/-}$ mice have only scattered $VCAM\text{-}1^+$ stromal cells resident in the lamina propria of the small intestine. This indicates that VCAM-1 expression of intestinal stromal cells is preserved even when IL-$7R\alpha^+$ $CD4^+CD3^-$ cell numbers in the intestine of $LT\alpha^{-/-}$ mice are strongly reduced. Importantly, in the absence of activated $\beta1$ integrin almost no or significantly reduced VCAM-1 cluster formation occurs, arguing for a direct role of $\alpha4\beta1$ integrin-expressing $CD4^+CD3^-$ cells in cluster formation of stromal cells.

The results are summarized in Figure 7. Initially, $CD4^+CD3^-$ hematopoietic cells enter the fetal intestine via a mechanism independent of $\alpha4\beta1$ integrin. The presence of activated $\beta1$ integrin on the surface of $CD4^+CD3^-$ hematopoietic cells allows interaction with CXCL13-secreting $VCAM\text{-}1^+$stromal cells. Whether $\beta1$ integrin has to be preactivated at this stage or can be activated in situ by CXCL13 was not addressed in this study. CXCL13 locally produced by stromal cells is likely to maintain $\alpha4\beta1$ integrin-activation and cellular attachment. CXCR5 also induces $LT\alpha1\beta2$-expression on $CD4^+CD3^-$ cells allowing $LT\beta R$-crosslinking on intestinal stromal cells and exchange of PP induction signals during the fetal/neonatal period.

We describe the feedback loop between CXCL13-secreting intestinal stromal cells and $CXCR5^+$ hematopoietic cells, hence reinforcing cellular attachment among both cell types. Intriguingly, $\alpha4\beta1$ integrin can activate in *cis* other cellular receptors such as $\alpha L\beta2$ (Rose et al., 2001). Since $VCAM\text{-}1^+$ stromal cells of fetal intestine express the corresponding receptor ICAM-1 (Hashi et al., 2001; Honda et al., 2001), it seems likely that additional feedback loops among $CD4^+CD3^-$ cells and stromal cells exist. Finally, our findings suggest that chemokine-driven activation of $\alpha4\beta1$ integrin could also

be important later in life when lymphoid structures are induced in chronic inflammation.

Experimental Procedures

Mice and Cell Preparation

Six- to seven-week-old C57BL/6 mice were purchased from Harlan Olac Ltd. (Bicester, United Kingdom). CXCR5$^{-/-}$ mice described by Förster et al. (1996), and LT$\alpha^{+/-}\beta^{+/-}$ mice obtained from R. Flavell (Koni and Flavell, 1998) were bred and maintained in the ISREC animal facilities on a B6×129 strain background. LT$\alpha^{-/-}$ mice were originally from De Togni et al. (1994). Pregnant mice were injected at E12.5 with 500 μg of rat mAbs specific for murine integrins or rat IgG as control. Injections were repeated every 3 days.

Antibodies and Cell Sorting

The Abs used in flow cytometry included anti-CD3 (17A2, PharMingen, San Diego, CA), anti-CD4-Ab (GK 1.5, PharMingen), and anti-rat IgG (polyclonal serum, Caltag) directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), cychrome (Cy), or allophycocyanin (APC). The following Abs were biotinylated: anti-β7 (M293, PharMingen), anti-β1 (HA25, PharMingen), and anti-α4 (PS/2). These Abs were visualized with Streptavidin conjugated to PE (Caltag, San Francisco, CA), Cychrome (PharMingen), or APC (Molecular Probes, Leiden, The Netherlands). Unconjugated Abs were anti-α4β7 (DATK 32), anti-β1$_{act}$ (9EG7), anti-α4 (PS/2; R1.2), anti-VCAM-1 (MK2/7), and anti-murine CXCR4 (2B11, Schabath et al., 1999) visualized by anti-rat Ig FITC. Lymphocytes were preincubated with anti-FcγRII mAb (2.4G2) whole supernatant and stained in one step as described (Finke and Acha-Orbea, 2001). Dead cells were excluded from acquisition by FSC/SSC gating. Data were analyzed with CELLQuest Software (Becton Dickinson Immunocytometry System, San Jose, CA). For sorting CD4$^+$CD3$^-$ cells, splenocytes from E18.5-d0 C57BL/6 mice were stained with a combination of anti-CD3 (FITC) and anti-CD4 (PE) and sorted with a FACStar + flow cytometer (Becton Dickinson & Co).

Adoptive Cell Transfer

Newborn (d0.5) CXCR5$^{-/-}$ mice were irradiated in two doses of 200 Rad each, 3 hr apart. 2×10^4 FACS-sorted CD4$^+$CD3$^-$ splenocytes were i.p. injected into newborn CXCR5$^{-/-}$ mice. Alternatively, 10^6 B lymphocytes from adult WT mice, WT IL-7Rα$^+$ fetal liver cells, CXCR5$^{-/-}$, IL-7Rα$^{-/-}$, or LTα$^{-/-}$ total spleen cells were injected. The presence of PP was determined by anti-VCAM-1 mAb (CD106, clone 429, rat IgG2a, PharMingen) immunohistochemistry of whole small intestine 2 to 6 weeks after reconstitution.

Immunohistochemistry

Whole small intestine was removed from newborn (d1-2) or 3- to 6-week-old CXCR5$^{-/-}$, LTα$^{-/-}$, or C57BL/6 mice. For VCAM-1 staining, tissues were immediately embedded in Tissue Tek OCT compound (Miles, Elkhart, IN) and snap-frozen in 2-methylbutane (Merck, Glattbrugg, Switzerland). Every fifth 7 μm cryostat section was fixed in cold acetone, frozen at −70°C, and re-fixed in acetone. After rehydration with PBS and blocking of nonspecific background staining with 5% goat serum and 1% BSA in PBS, tissue sections were incubated with anti-VCAM-1 mAb (429) for 1 hr at room temperature. Sections were then washed with PBS and incubated with peroxidase-conjugated goat-anti rat IgG (TagoImmunologicals, Camarillo, CA) for 45′. Bound peroxidase activity was developed using 3-amino-9-ethylcarbazole (Fluka, Buchs, Switzerland). At least 40 consecutive sections of the whole intestine were analyzed by VCAM-1 mAb staining. In order to detect HEV on frozen tissue, anti-mouse MAdCAM-1 mAb (rat IgG2a; Meca 89) or the anti-PCAM-1 mAb (rat IgG2a, CD31, MEC13.3, PharMingen)-biotinylated was used, and for visualization of the Ab binding either peroxidase-conjugated goat anti-rat IgG or peroxidase-conjugated Streptavidin was used. Sections of spleen, MLN, and PP isolated from mice treated with either control Ab (polyclonal rat IgG) or mAb specific for β1 integrin (9EG7), α4 integrin (PS/2), α4β7 integrin (DATK 32), or VCAM-1 (MK2/7) were incubated with rat anti-mouse CD4 (H129), washed with PBS, and incubated with goat anti-rat peroxidase (HRP, TAGO). Antibody binding was visualized by incubation with HRP-substrate conjugated to DAB (Sigma). In a second step, sections were washed and incubated with either biotin-conjugated rat anti-mouse B220 (RA3-6B2, Caltag) or biotin-conjugated hamster anti-mouse CD3ε (145-2C11, PharMingen). After incubation with Streptavidin alkaline phosphatase (AP, Boeringer, Germany), Ab binding was visualized with AP substrate conjugated to fast blue (Sigma).

Whole mount immunohistochemistry was performed as described (Hashi et al., 2001). In brief, small intestines from d2 C57BL/6 mice were separated from serosa and fixed in 4% paraformaldehyde solution for 30 min at 4°C. After quenching free aldehyde groups with 4% glycine in PBS for 30 min at 4°C, specimens were dehydrated by incubation in 50, 70, and 100% methanol in PBS at 4°C. To block endogenous peroxidase, the specimens were incubated for 30 min at room temperature in methanol: 30% H_2O_2 20:1. The intestines were then blocked by incubation twice in PBSMT (1.5% skim milk and 0.1% Triton X-100 in PBS) for 1 hr at room temperature, and overnight incubated with PBSMT containing anti-VCAM-1 (mAb 429). After washing five times in PBSMT at 4°C, the primary mAb was detected by incubating with HRP-conjugated anti-rat Ig Ab (TAGO) overnight at 4°C. After washing in PBS and TrisHCl (0.05 M [pH 7.6]), antibody binding was visualized by incubation with HRP-substrate conjugated to DAB (Sigma).

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